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Ultrastructure of Cuticular Exudates and Related Cuticular Changes on Juveniles in *Heterodera glycines*[1]

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ABSTRACT: Fibrillar exudates formed on the cuticle surface of parasitic *Heterodera glycines* second-stage juveniles (J2) during feeding on soybean roots. Accumulation of cuticular exudates was correlated with the fibrillar and porous nature of the epicuticle, exocuticle, and endocuticle. The apparent source of the exudates was the hypodermis, where coalesced secretory vesicles were assembled by Golgi bodies and transferred to the inner surface of the apical membrane of the hypodermis. Products of the secretory vesicles were apparently released into a secretion accumulation zone at the base of the endocuticle by some mechanism and then extruded through and onto the cuticle surface. Golgi bodies occurred in large expanded regions of the hypodermis, especially in the hypodermal cords, where prominent nuclei and other cellular components were located. During ecdysis of the J2 cuticle and early stages of third-stage juvenile (J3) cuticle formation, fine reticulate material accumulated at the secretory–excretory pore. Concurrently, moderately electron-dense material occurred in the invaginated cephalic region and in the space extending between the molted J2 cuticle and the entire J3 body.

KEY WORDS: cuticle, cuticular exudations, exudations, *Heterodera glycines*, ultrastructure.

Thick cuticular exudates on the surface of adult females of the sugarbeet cyst nematode, *Heterodera schachtii* Schmidt, 1871, were named "subcrystalline layer" by Schmidt (1871, 1872). The layer was thought to be a by-product that was produced by an outside organism such as a fungus (Brown et al., 1971). This concept has been altered because exudates are produced on the cuticle of the same species feeding on host plants grown under monoxenic culture conditions (Zunke, 1985). Recent ultrastructural studies of second- (J2) and third-stage juveniles (J3) of *H. schachtii* support the concepts that the exudate is produced by the nematode alone and that the cuticle is a relatively porous structure providing continuity between secretory granules in the hypodermis and fibrillar exudates on the cuticle surface (Endo and Wyss, 1992).

This study was initiated to elucidate formation of cuticle exudations in the soybean cyst nematode, *Heterodera glycines* Ichinohe, 1952, and compare the results to those obtained for *H. schachtii*.

Materials and Methods

Infective and parasitic stages of *H. glycines* in infected soybean (*Glycine max*) roots were prepared for electron microscopy by previously described procedures (Endo and Wergin, 1973; Wergin and Endo, 1976). Briefly, seedlings of the susceptible cultivar Lee were raised in vermiculite and inoculated with infective J2 of races 3 and 4 of the soybean cyst nematode. Nematode-infected root segments from several experiments were periodically sampled within 5 hr to 7 days after inoculation (DAI). Infective J2 and root samples were fixed in buffered 3% glutaraldehyde (0.05 M phosphate buffer, pH 6.8) at 22°C for 1.5 hr, washed for 1 hr in 6 changes of the same buffer, postfixed in 2% osmium tetroxide in the same buffer for 2 hr, dehydrated in an acetone series, and infiltrated with a low-viscosity medium (Spurr, 1969). Silver-gray sections were cut on an ultramicrotome with a diamond knife and mounted on uncoated 75×300-mesh copper grids. The sections were stained with uranyl acetate and lead citrate and viewed in a Philips 301 or 400T electron microscope operating at 60 kV with a 20-μm objective aperture.

Results

The cuticle of infective J2 of *H. glycines* consists of 3 distinct zones: an outer epicuticle, an underlying exocuticle, and a striated endocuticle in contact with or adjacent to the hypodermis (Figs. 1, 2). The epicuticle is thin with closely arranged electron-dense fibrillar striations. The exocuticle is moderately electron-dense with fine fibrillar strands that appear to traverse the epicuticle and show continuity through a flocculent intermediate zone with the striated endocuticle (Fig. 2). Hemidesmosomes are arranged circumferentially and usually in pairs under each cuticle annulus (Figs. 1, 2). The hemidesmosomes connect the base of the endocuticle to the basal la-

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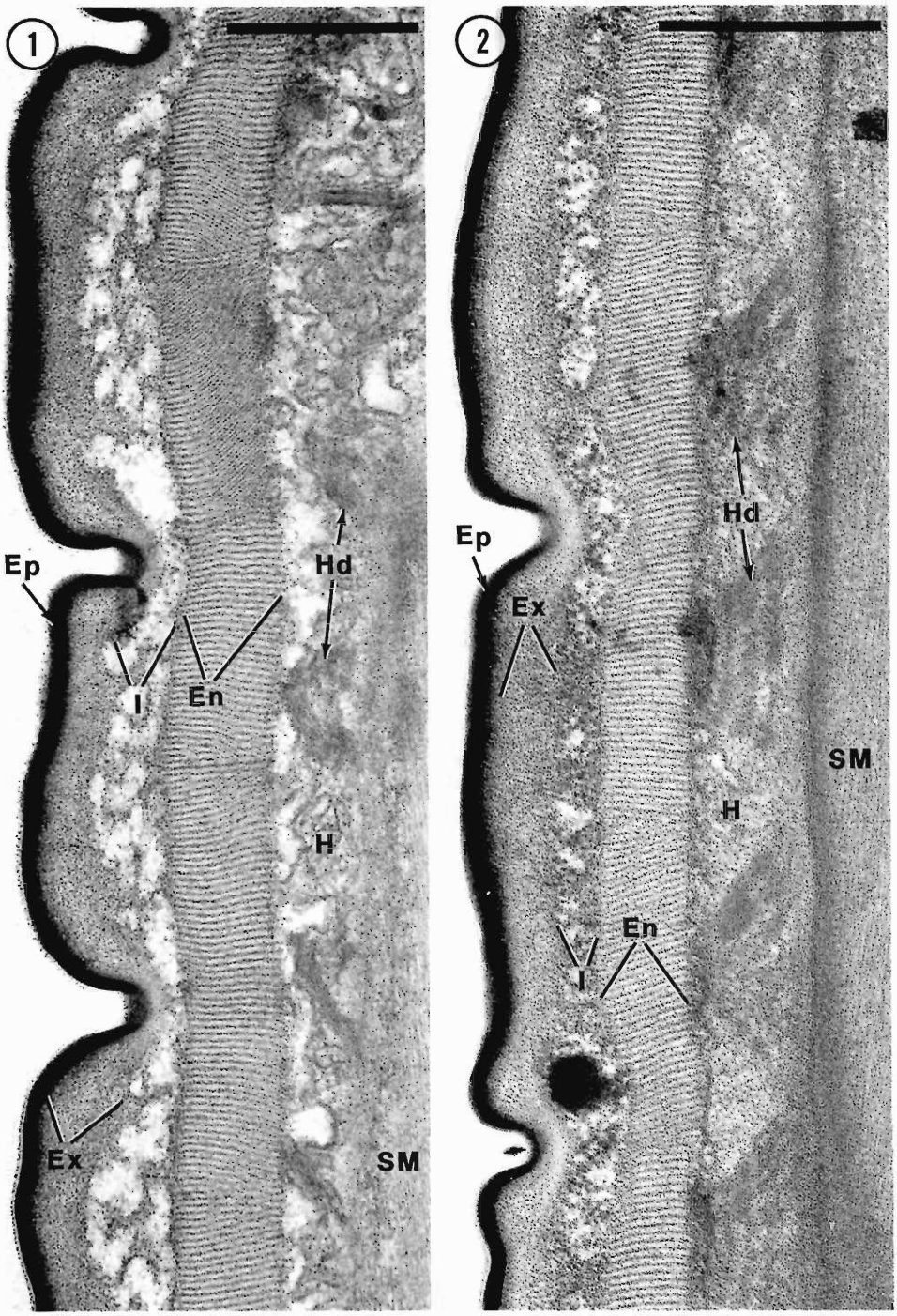
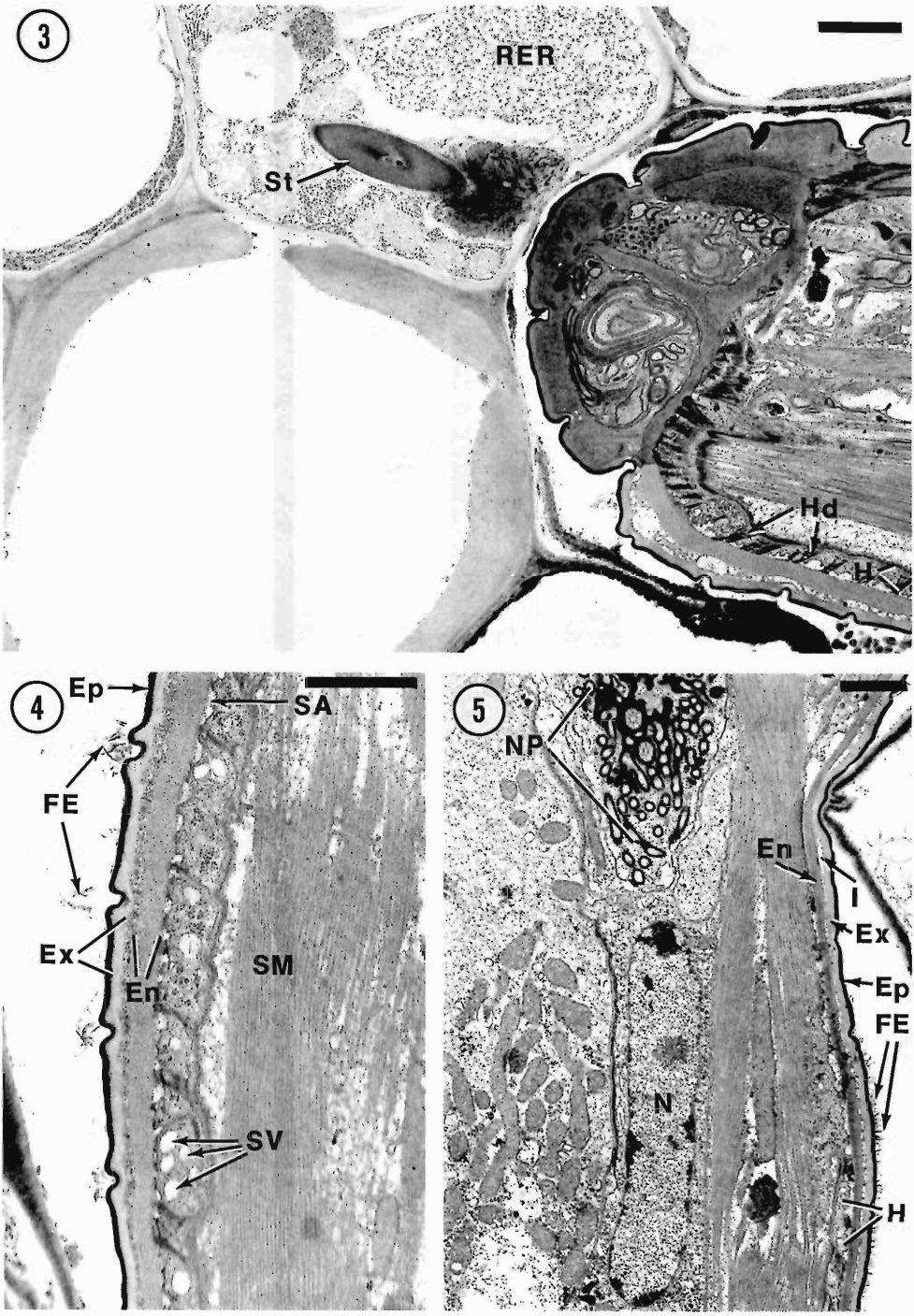


Figures 1, 2. Longitudinal sections through infective and parasitic J2 of *Heterodera glycines* 5 hr after inoculation of soybeans roots. 1. Section of infective J2 posteriad from lip region showing rounded annulations of cuticle with flocculent intermediate zone (I) between the exocuticle (Ex) and endocuticle (En). Hemidesmosomes (Hd) appear in pairs under each annulation. Ep, epicuticle; SM, somatic muscles. 2. Fibrils within exocuticle (Ex) of J2 appear similar in dimensions to striae of endocuticle (En). Hemidesmosomes (Hd) appear uniformly stretched between endocuticle and the somatic muscles (SM) near the amphidial cell region. H, hypodermis. Bars = 0.5 μm.



Figures 3–5. Cuticle exudates and related secretory vesicles in hypodermis of *Heterodera glycines* J2, 1 day after inoculation. 3. Lip region of J2 with stylet (St) extended into initial syncytial cell located adjacent to metaxylem vessel. Hemidesmosomes (Hd) stretched within expanded hypodermis (H). RER, rough endoplasmic reticulum. 4. Longitudinal section near the stylet base shows fibrillar exudations (FE) concentrated at annulations of cuticle. Electron-transparent secretory vesicles (SV) occur throughout cytoplasm of hypodermis. En, endocuticle;

mella of the somatic musculature in the intercordal regions of infective (Fig. 1) and parasitic J2 (Fig. 2). The hypodermis adjacent to the somatic muscles is narrow in these regions but increases in width and volume as it extends into the body cavity at the dorsal, ventral, and lateral cord sectors. In hemidesmosome-free areas, the base of the endocuticle is either directly contiguous with the apical membrane of the hypodermis or in contact with secretory vesicle deposition sites that are especially evident after host penetration and initiation of feeding. At 5 hr after inoculation, fibrillar continuity between the zones of the cuticle is present, but no exudations from the cuticle surface are apparent (Fig. 2).

Day 1

Initial signs of cuticle exudations are present in specimens located at feeding sites where syncytial cells have been stimulated (Fig. 3). While exudations are obvious at the annulations (Fig. 4), some specimens show similar exudations directly on the annulus surface (Fig. 5). The intermediate zone between the exocuticle and the endocuticle is electron-transparent and appears similar to the zone observed in infective J2 (Fig. 1). The expanded underlying hypodermis contains electron-transparent secretory vesicles, whose products presumably enter the secretion accumulation sites that lie between the membrane of the hypodermis and the base of the endocuticle (Fig. 4).

Day 2

Transverse or longitudinal sections through the anterior of parasitic J2 show moderate accumulations of fibrillar exudations on the cuticle surface (Figs. 6, 7). Fibrillar exudates extend through the endocuticle, exocuticle, and epicuticle (Fig. 7, inset). The intermediate zone between the exocuticle and endocuticle shows increased density but retains some of the flocculent features observed in the preparasitic J2 (Fig. 2). Fibrillar exudations are oriented perpendicular to the body surface (Figs. 6, 7), except where the body is appressed against the host tissue. Within the hypodermis, Golgi bodies assemble secretory vesicles that coalesce and appear as electron-transparent secretory granules close to the apical membrane of the hypodermis. The contents of the secretory granules may be released into a secretion accumulation zone located between the hypodermis and the endocuticle (Fig. 7).

Day 3

Fibrillar exudates of the cuticle surface of parasitic J2 at 3 DAI are relatively dense (Figs. 8, 9). Fibrillar continuity between the exudates and the various regions of the cuticle is discernible. The hypodermis associated with the cuticle at 3 DAI contains electron-transparent secretory vesicles that apparently arise from Golgi bodies (Fig. 9).

Day 4

At 4 DAI, parasitic J2 in early stages of molting show electron-dense accumulations between the J2 cuticle and the outer boundary of the J3 body (Figs. 10, 11). During molting, the dissolution of portions of the most anterior region of the J2 results in the separation of the J2 cuticle with the attached stomatal wall and stylet cone from the newly formed boundaries of the J3 (Fig. 10). The electron-dense zone under the cuticle at the anterior of the J2 merges with an accumulation of similar material found in the invaginated anterior of the J3. Thus, the former apical membrane of the J2 hypodermis becomes the precursor of the J3 cuticle (Fig. 11). During early stages of molt, the hypodermis of the J3 contains electron-dense and -transparent secretory vesicles associated with earlier exudate formation and molting (Fig. 11).

Day 6

At 6 DAI, the J2 cuticle becomes detached from the body surface of the J3 and the moderately electron-dense material separating the J2 cuticle and the J3 body (Figs. 12–14). All zones of the J2 cuticle and fibrillar exudations remain intact (Figs. 13, 14). In contrast to the coarse, moderate to electron-dense depositions in the invaginated anterior (Fig. 12) and the narrow space between the molting J2 cuticle and the J3

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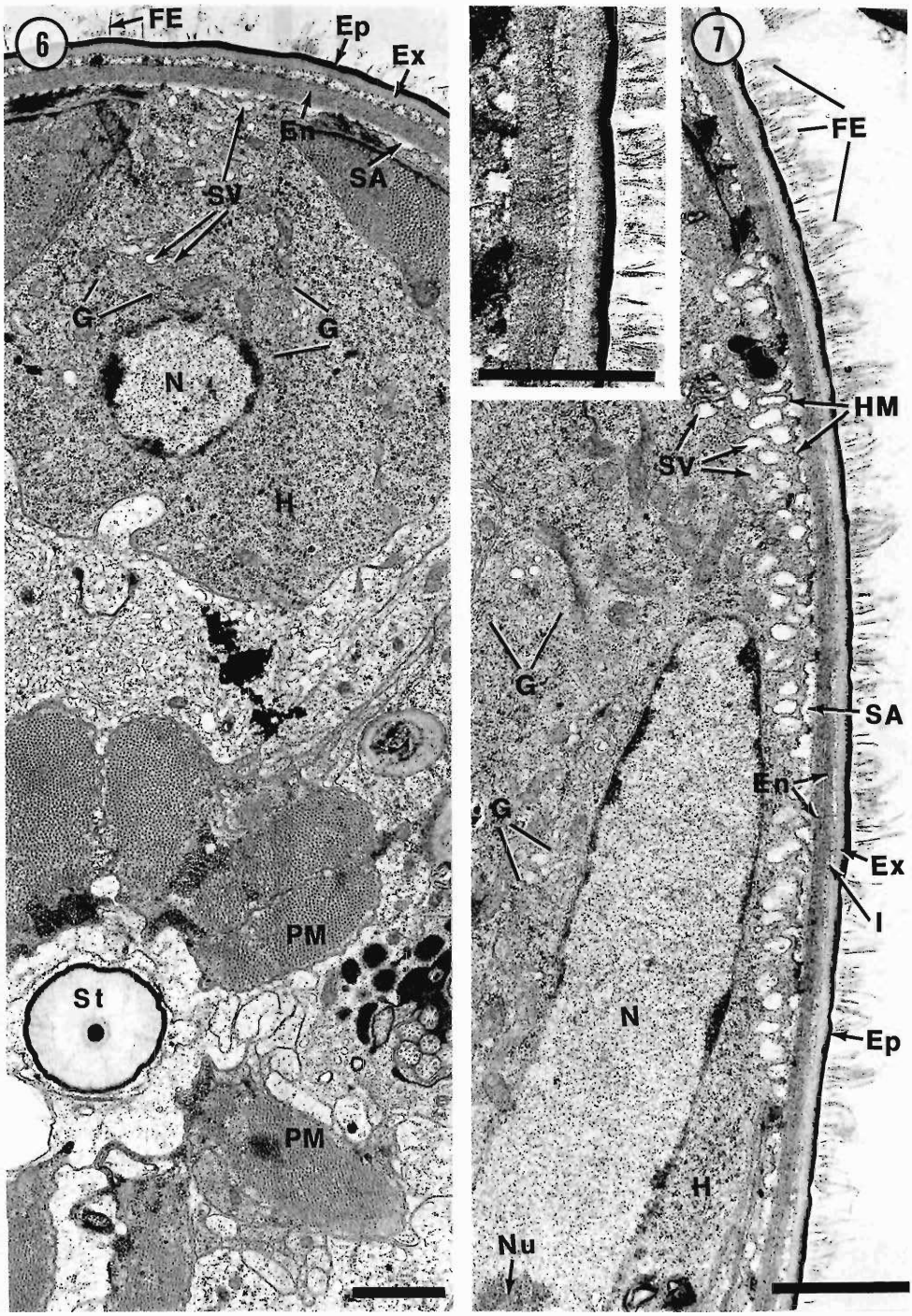
Ep, epicuticle; Ex, exocuticle; SA, secretion accumulation zone; SM, somatic musculature. 5. A third specimen shows fibrillar exudations (FE) along annules of cuticle. H, hypodermis; N, nucleus; NP, nerve process. Bars = 1.0 μm.



Figures 6, 7. ***Heterodera glycines*** **J2, 2 days after inoculation. 6. Transverse section in region of stylet shaft shows sites of Golgi (G) and secretory vesicles (SV) in hypodermis (H). Vesicles tend to accumulate near apical membrane of hypodermis, where their contents are apparently transferred to secretion accumulation zone (SA) between plasmalemma and base of endocuticle (En). Fibrillar exudates (FE) accumulate on surface of epicuticle (Ep). Ex, exocuticle; N, nucleus; PM, protractor muscles; St, stylet. 7. Fibrillar exudates (FE) present on cuticle surface near stylet region of longitudinal section of parasitic J2. Section through hypodermal chord shows**

body surface (Fig. 14), a fine reticulate material is present at the duct terminus of the secretory–excretory (S-E) gland (Fig. 13). Electron-dense depositions on the outer surface of the J3 hypodermal membrane constitute the very early stages of J3 cuticle formation. The area of low electron density between the trilaminar hypodermal membrane and the adjacent electron-dense depositions accounts for the bilayered appearance of the initial stage of the J3 cuticle (Fig. 14, inset).

In other specimens at 6 DAI, the J2 cuticle is completely separated from the J3 body. Although the various zones of the J2 cuticle are discernible, deterioration of the exocuticle and endocuticle has taken place (Figs. 15–18). Some variation in the rate of development existed among the J3 specimens observed at 6 DAI. However, most of the nematodes had completed the molting process and new stylet components were well developed. The original electron-dense material accumulated during early stages of molt at about 4 DAI has become electron-transparent and diffuse. The material is distributed throughout the space formed by the expanded deteriorating J2 cuticle and developing J3 body (Figs. 15–18). Prior to the complete separation of the J2 cuticle, the fine particulate material that accumulated near the outlet of the S-E duct at 5 DAI appears diffuse and retains sections of the S-E duct wall of the J2 apparently extruded during molting (Figs. 16, 17). At sites distant from the base of the S-E gland pore, less concentrated material was distributed between the J2 cuticle and the J3 body surfaces. These accumulations occurred at the time when the epicuticle was being formed and at later stages when all zones of the J3 cuticle were deposited, including the exocuticle, endocuticle, and basal layer that adjoins the hypodermis. At these later stages of J3 cuticle development, an S-E gland cell shows sections of the J2 duct wall lying within sections of a J3 duct wall (Fig. 16).

Day 7

Within 7 DAI, the J2 cuticle is disintegrated with only the epicuticle and parts of the stylet cone discernible (Figs. 19, 20). The same cuticle zones are present as in the J2, and an additional basal layer appears. The J3 stylet, supported by a stomatal wall, appears to be in a developmental stage preparatory to host penetration (Fig. 19). However, since feeding has not commenced, cuticular exudations are lacking. The cephalic region of the J3 has a stylet formed (Fig. 19) well beyond the initial components that usually occur in the invaginated anterior of early stages of J3 development. The cuticle of the anterior region is highly convoluted and of low electron density (Fig. 19). In the dorsal gland region of the J3, the cuticle has a relatively thick flocculent basal layer (Fig. 21).

Discussion

The occurrence of exudates on the surface of the sugarbeet cyst nematode, *H. schachtii,* while feeding on root tissues of *Raphanus sativus* var. *oleiformis* under monoxenic cultural conditions (Zunke, 1985), established that cuticle exudates are produced in the absence of an external factor such as a fungus. Such a microorganism was proposed as a symbiont for the formation of a subcrystalline layer observed on adult females of *H. schachtii* by Schmidt (1871, 1872) and on other species including *H. mani, H. avenae,* and *H. trifolii* (Brown et al., 1971). The subcrystalline layer on the cuticle surface of the latter 3 adult cyst nematodes consisted of even-numbered saturated fatty acids and their calcium salts; the layer was considered to be a metabolic product of an unidentified fungus that had fed on the secretions of the nematodes. The subcrystalline layer was proposed to function as a barrier to potential pathogens and predators (Brown et al., 1971). The cuticle of a parasitic J2 of *H. glycines* was reported to have a uniform layer of fibrillar material that was oriented perpendicular to its surface (Endo, 1987). Fibrillar exudates on the cuticle of sedentary J2 of *Globodera rostochiensis* emerged from annulations (Forrest et al., 1989).

Recent ultrastructural studies of *H. schachtii* in *Brassica* sp. roots by Endo and Wyss (1992) showed that cuticle exudates occurred within 1 day after inoculation. Three to 4 days later, ex-

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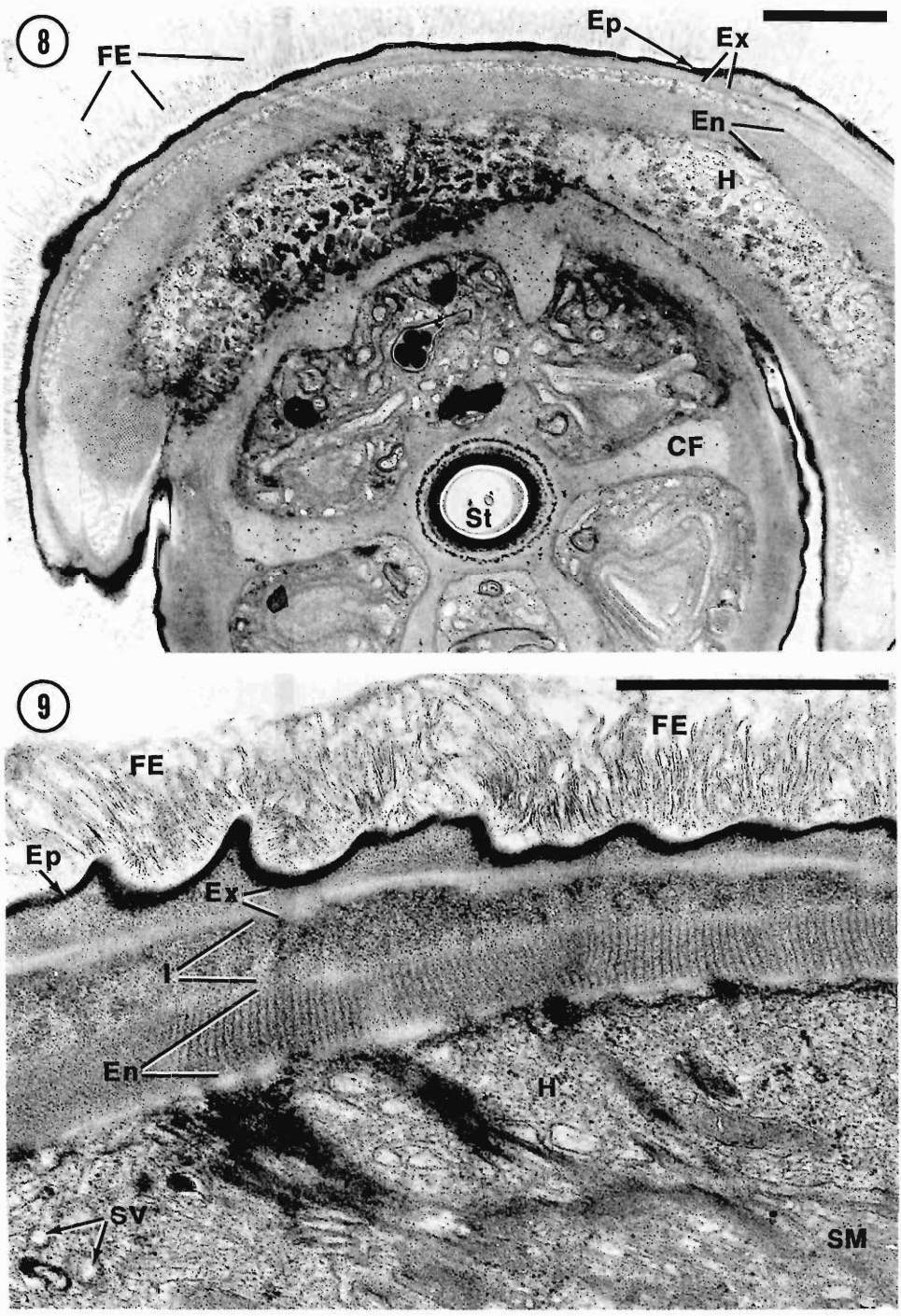
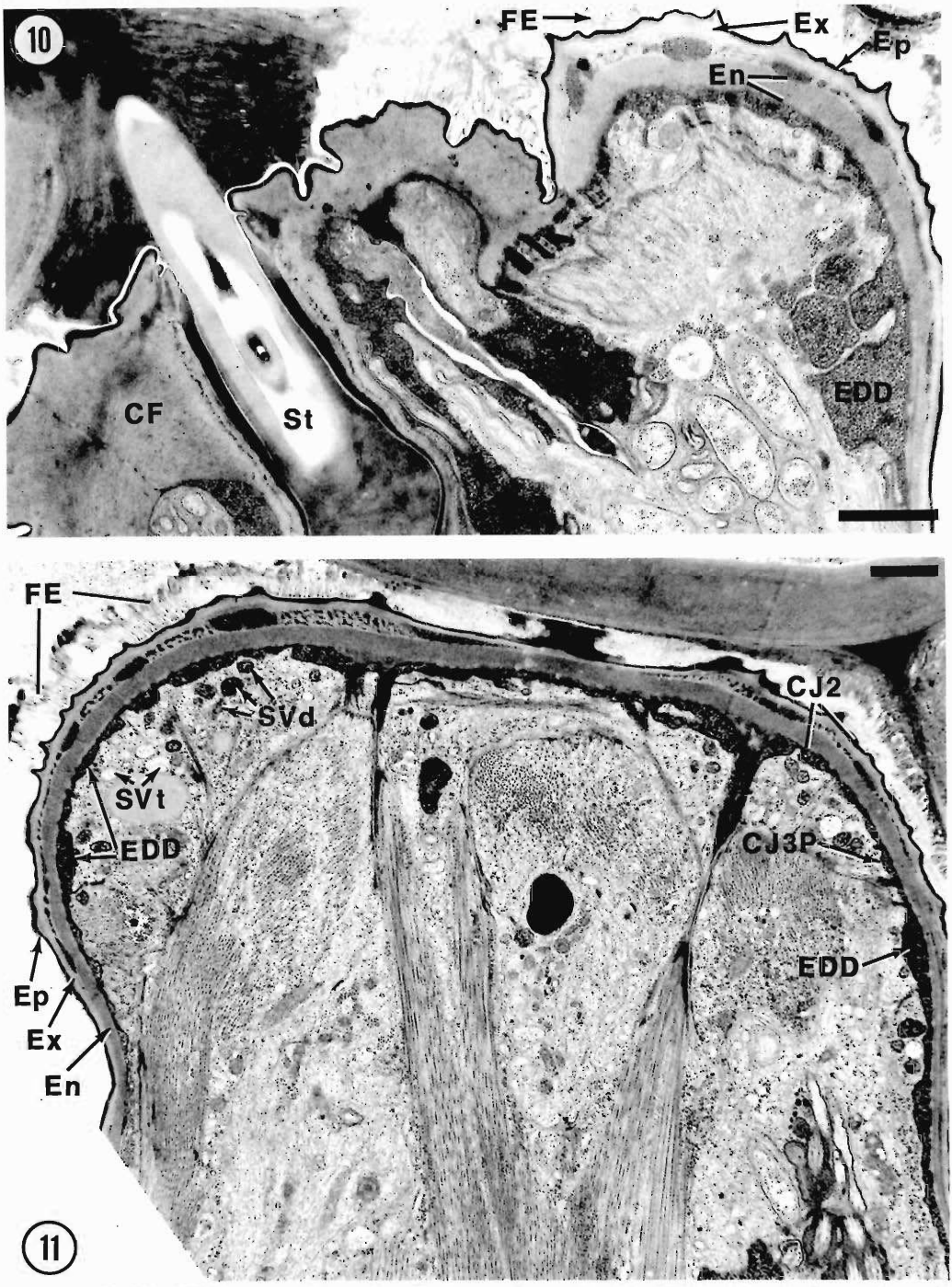
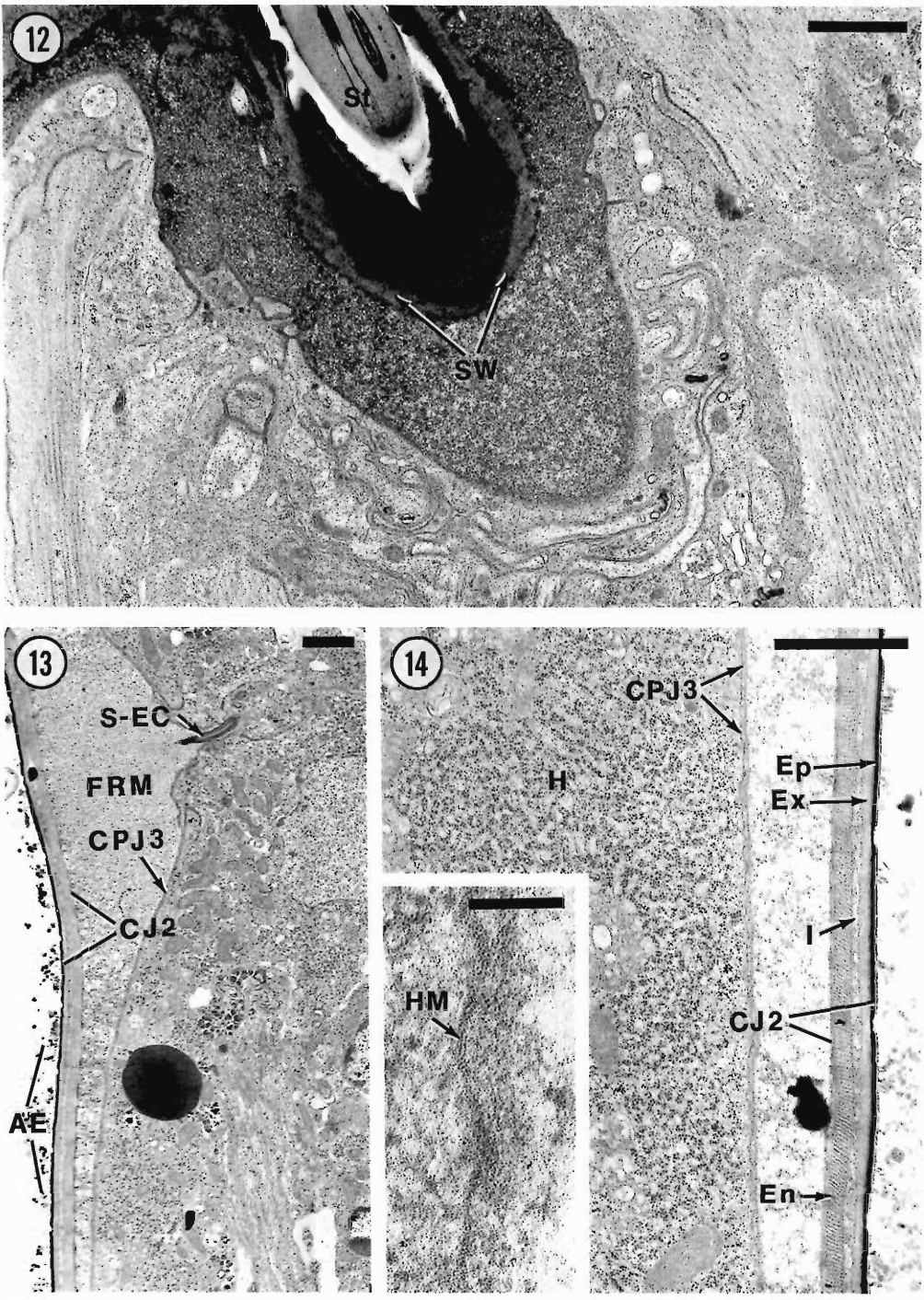
elongated nucleus (N) and extensive accumulation of secretory vesicles (SV) near Golgi (G) and apical membrane (HM) of the hypodermis (H). En, endocuticle; Ep, epicuticle; Ex, exocuticle; I, intermediate zone; Nu, nucleolus; SA, secretion accumulation zone. Bars = 1.0 μm. Enlargement shows fibrillar exudate continuity with portions of the cuticle. Bar = 0.5 μm.



Figures 8, 9. Exudates on cuticle of parasitic J2, 3 days after inoculation. 8. Cross-section immediately posteriad from cephalic region shows fibrillar exudates (FE) on cuticle surface. CF, cephalic framework; En, endocuticle; Ep, epicuticle; Ex, exocuticle; H, hypodermis; St, stylet. 9. Longitudinal section near the cephalic region of J2 shows dense accumulations of fibrillar exudates (FE) over entire cuticle. En, endocuticle; Ep, epicuticle; Ex, exocuticle; H, hypodermis; I, intermediate zone; SM, somatic musculature; SV, secretory vesicles. Bars = 1.0 μm.



Figures 10, 11. Cuticle separation and early stages of second molt of *Heterodera glycines*, 4 days after inoculation. 10. Longitudinal section shows electron-dense deposits (EDD) occurring between J3 endocuticle and developing outer boundaries of J3. Stylet cone (St) retained within the stomatal wall and cephalic framework (CF). Residual fibrillar exudates (FE) present on second-stage cuticle. En, endocuticle; Ep, epicuticle; Ex, exocuticle. 11. Tangential section below that of Figure 10 shows outlines of transparent (SVt) and electron-dense (SVd) secretory vesicles near apical membrane of hypodermis. Residual fibrillar exudate (FE) adheres to J2 cuticle. CJ2, cuticle of J2; CJ3P, cuticle of CJ3 primordium; EDD, electron-dense deposits; En, endocuticle; Ep, epicuticle; Ex, exocuticle. Bars = 1.0 μm.



Figures 12–14. Ecdysis of parasitic *Heterodera glycines* J2, 6 days after inoculation. 12. Entire anterior mid-central portion of J2 has deteriorated. Stylet cone (St) and stomatal wall (SW) are retained in contact with J2 cuticle. Tissues at base of invaginated anterior is site of J3 stylet initials (Endo, 1985). 13. Longitudinal section through secretory–excretory gland showing accumulation of fine reticulate material (FRM) concentrated at the pore. Remnants of fibrillar exudates, amorphous cuticular exudates (AE) adhere to J2 cuticle. Outer boundary

tensive accumulations were present on the annuli and, to a limited extent, near the annulations. The exudations appeared as fibrils that extended through the endocuticle, intermediate zone, exocuticle, and epicuticle. Endo and Wyss (1992) proposed that secretion vesicles, assembled at many Golgi sites in the hypodermis of *H. schachtii,* coalesced and formed large electron-translucent vesicles in the cytoplasm. These vesicles appeared to migrate toward the cuticle, where they would contact the plasmalemma and transfer their contents to an accumulation site by exocytosis or a similar mechanism.

Based on light microscopic (Zunke, 1985; Wyss and Zunke, 1986; Wyss et al., 1986; Wyss, 1992) and ultrastructural (Endo and Wyss, 1992) observations on feeding and cuticle exudations in *H. schachtii,* the cuticular fibrillar material of *H. glycines* can also be designated as cuticular exudate. Although video-enhanced contrast light micrographs of in vivo activity of *H. glycines* in host roots are not available, the exudations appear to correlate with the feeding periods of the nematode. For example, exudations occurred on the cuticle of parasitic J2 at 3 DAI, when stylet tips of these specimens were observed in syncytia containing feeding tubes (Endo, 1987, 1991). The abundance of secretory granules and the presence of numerous sites of Golgi activity in the hypodermis are indicative of active metabolism of the nematode. In an ultrastructural study of cuticle formation in *Meloidogyne javanica,* Bird and Rogers (1965) showed that at the start of molting the hypodermis becomes thickened and filled with ribosomelike granules that are probably associated with the formation of a new cuticle. Recently, it was observed that *H. glycines* grown in monoxenic culture in the absence of any other organism produced exudations 15 DAI (Endo, unpubl.).

Information is lacking on the sequence of events that influence the formation and localization of secretory vesicles in the hypodermis as they accumulate below the endocuticle and pass through other zones of the cuticle to emerge as exudates. Visualization of sites of synthesis and movement of secretory vesicles may be enhanced by utilizing labeling techniques such as fluorescence microscopy and autoradiography. In monoxenic cultures of various cruciferous plants, exudates from *H. schachtii* failed to show carbohydrate binding of fluorochrome-conjugated lectins; however, some binding may have been associated nonspecifically with fatty acids. Aumann et al. (1991) concluded that exudates may function as a protective layer for the cuticle and mask recognition by root tissues.

Involvement of the S-E gland in ecdysis was previously reported in the animal parasitic species, *Phocanema decipiens* (Davey and Kan, 1968), and in plant parasitic species (Nakasono, 1973; Bird, 1984). Observations of *Rotylenchulus reniformis* showed swelling in the region of the excretory pore of the J2 during the start of the second molt and seemed to implicate the excretory system in the early stages of molting. The excretory or S-E system (Nelson et al., 1983; Bird and Bird, 1991) was also implicated at a later stage after the final molt, when the duct wall had thickened and appeared to extrude material under the shed cuticles. The material was suggested to be remnants of the cuticular lining of the duct or associated with physiological functions related to molting (Bird, 1984). In the same species, Nakasono (1973) had observed dilations in the excretory gland at the final molt. Wright and Perry (1991) failed to observe changes or activities in the excretory system during molting of *Aphelenchoides hamatus.* Instead, just prior to ecdysis, the metacorpus was very active.

The transition from J2 to J3 of the soybean cyst nematode is accompanied by fundamental changes in the accumulation of electron-dense material in the invaginated anterior region of the developing J3 (Endo, 1985). During molt, the boundary of the electron-dense zone merges with

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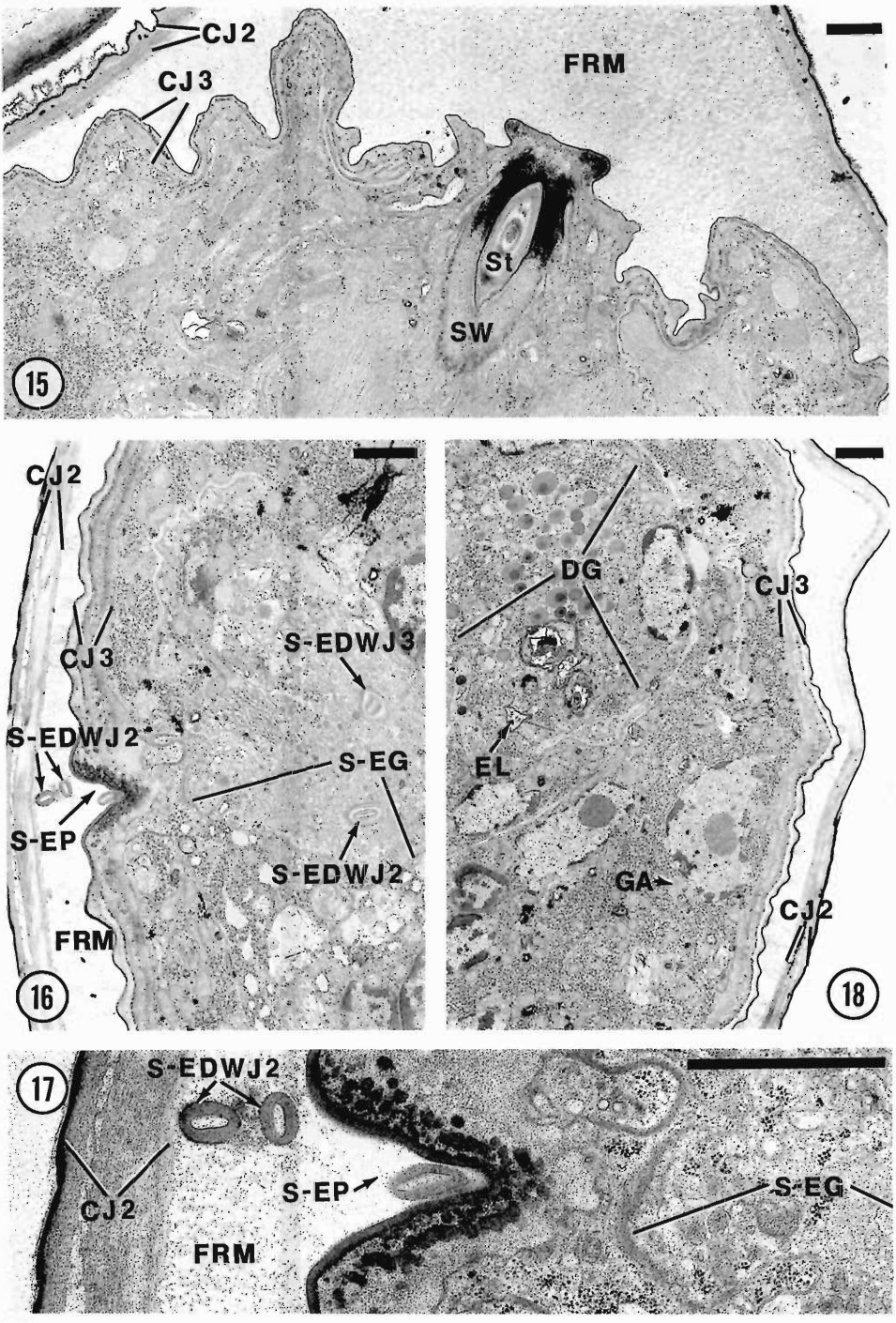
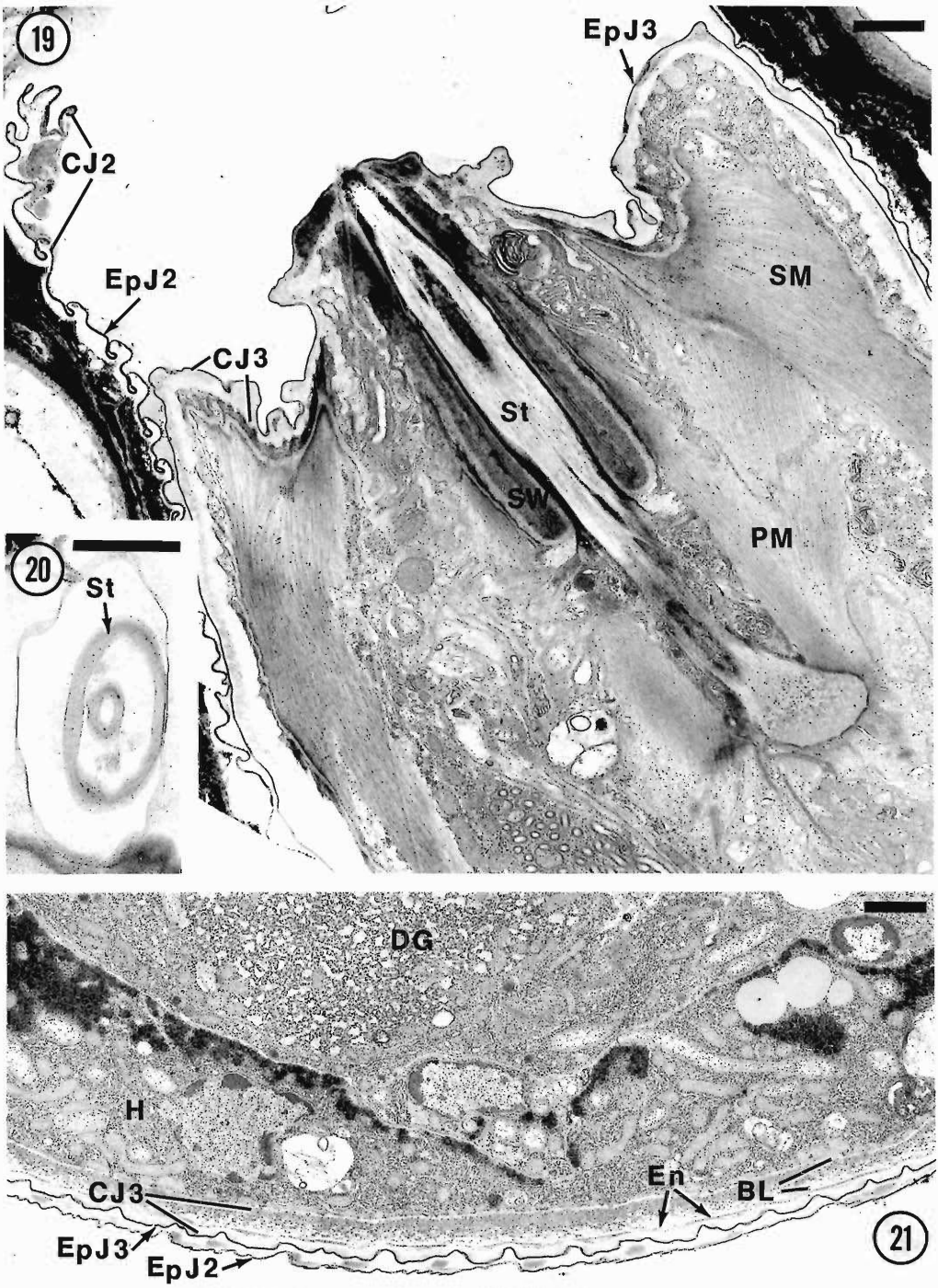
of J3 consists of trilaminar membrane from which short fibrillar projections appear to form on a primordial J3 cuticle (CPJ3). CJ2, cuticle of J2; S-EC, secretory–excretory canal. 14. Cuticle of J2 (CJ2) separated from J3 at some distance away from the secretory–excretory pore. Longitudinal section through apical membrane of hypodermis reveals a trilaminar membrane with short projections similar in appearance to striae at the base of the J2 endocuticle. Space between the J2 cuticle and the J3 body is filled with reticulate material but is dissimilar to the fine reticulate material accumulating near the secretory–excretory canal pore. CPJ3, cuticle primordium of J3; En, endocuticle; Ep, epicuticle; Ex, exocuticle; H, hypodermis; I, intermediate zone. Bars = 1.0 μm. Inset shows cuticle primordium of J3. HM, hypodermal membrane. Bar = 0.1 μm.



Figures 15–18. Longitudinal sections of J3 showing newly formed stylet, secretory–excretory pore site, and deteriorated J2 cuticle, 6 days after inoculation. 15. In contrast to earlier stages of J3 (Figs. 12–14) stylet (St) formation is well advanced and stylet is surrounded by thick stomatal wall (SW); J3 cuticle (CJ3) is multizoned. CJ2, cuticle of J2; FRM, fine reticulate material. 16. Section through secretory–excretory pore (S-EP) and gland (S-EG) shows segments of J2 secretory–excretory gland duct walls (S-EDWJ2) within the newly formed J3 duct wall (S-EDWJ3) or remnants of J2 duct wall accumulated between the J2 (CJ2) and J3 cuticles (CJ3). FRM, fine reticulate material. 17. Section of specimen in Figure 16 at higher magnification and different level shows part of secretory–excretory canal within the invaginated J3 cuticle which forms the secretory–excretory pore (S-EP). CJ2, cuticle; FRM, fine reticulate material; S-EDWJ2, secretory–excretory gland duct wall of J2; S-EG, secretory–excretory gland. 18. Sector of J3 shows well developed cuticle (CJ3) and detached convoluted *J2 cuticle (CJ2)* with signs of deterioration. DG, dorsal gland; EL, esophageal lumen; GA, Golgi apparatus. Bars = 1.0 μm.



Figures 19–21. Longitudinal and cross-sections of a J3 of *Heterodera glycines,* 7 days after inoculation. 19. Developing anterior of J3 with stylet (St) supported by stomatal wall (SW). Thick cuticle of J3 (CJ3) contrasts with deteriorated J2 cuticle (CJ2) consisting mainly of the epicuticle (EpJ2). EpJ3, epicuticle of J3; PM, protractor muscles; SM, somatic muscles. 20. Extreme anterior of J3 in Figure 19 shows nematode with residue of stylet cone (St). 21. Cross-section near dorsal gland (DG) region shows J3 cuticle (CJ3) with interrupted bands of narrow striated endocuticle (En) and thick basal layer (BL). Remaining J2 epicuticle (EpJ2) adjacent to J3 cuticle (EpJ3). H, hypodermis. Bars = 1.0 μm.

the widening zone between the J2 cuticle and the J3 body, which is bounded initially by the membranes of the hypodermal cells. Work is needed to determine the relationship between the reticulate material formed near the S-E terminus and the accumulated electron-dense material within the invaginated anterior of a developing J3. Because advanced stages of the J3 show J2 duct wall remnants within sectors of the J3 duct wall, morphology of the duct wall of the S-E canal is apparently related to the developmental stage of the J3 cuticle.

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